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1 **S₁₆ and T₁₈ mannosylation sites of LppX are not essential for its activity in**
2 **phthiocerol dimycocerosates localization at the surface of *Mycobacterium***
3 ***tuberculosis***

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21 **Abstract**

22 LppX is an important virulence factor essential for surface localization of phthiocerol
23 dimycocerosates (DIM) in *Mycobacterium tuberculosis*. Based on Concanavalin A recognition,
24 *M. tuberculosis* LppX (LppX-tb) was initially proposed to be glycosylated in *M. tuberculosis* and
25 more recently this glycosylation was characterized by mass spectrometry analysis on LppX-tb
26 expressed and purified from *Corynebacterium glutamicum*. Here, using this model organism
27 and *Mycobacterium smegmatis*, we show that S16 and T18 residues of LppX-tb are indeed
28 glycosylated with several hexoses units. Interestingly this glycosylation is strictly dependent
29 on the mannosyl transferase PMT which, in *M. tuberculosis*, has been reported to be crucial
30 for virulence. Using a site directed mutagenesis approach, we were able to show that the
31 absence of S16 and T18 glycosylation does not alter phthiocerol dimycocerosates (DIM)
32 localization in *M. tuberculosis*-

33

34 **Keywords** : *Corynebacterium glutamicum*, *Mycobacterium*, glycosylation, phthiocerol
35 dimycocerosates

36

37 1. Introduction

38 Among post-translational modifications of proteins, glycosylation is the most common
39 modification occurring in nature. These modifications which were initially thought to be
40 restricted to eukaryotic cells are now better characterized and known to be widely distributed
41 in bacteria and archaea. All these organisms share similar *N*-glycosylation and *O*-glycosylation
42 process. While *N*-glycosylation corresponds to the addition of glycosyl residues on asparagine,
43 *O*-glycosylation targets serine, threonine or tyrosine. A large variety of glycans (differing in
44 chain length, type of ose, chemical bonds and/or chemical modifications) might be added and
45 thus creating a vast repertoire of modifications contributing to protein diversity [1,2].

46 In eukaryotes numerous functions have been assigned to protein glycosylation such as
47 protein folding and stability, targeting to distinct cellular compartments and cell to cell
48 recognition [2]. Glycans can affect protein stability and accessibility as well as enable specific
49 interaction. Moreover the nature of the glycan is important as protein dynamics differs
50 depending on glycan chemistry [3]. As an example Prates et al. [3] studied the role of α -
51 mannosylation of a fungal Intrinsically Disordered Protein (IDP) by comparing different
52 glycosylated variants of its linker. Compared to the other tested glycans, α -mannose was
53 shown to increase proteolytic resistance, restrict protein flexibility and lead to more extended
54 conformations of this linker. According to the authors this would explain why, in fungi and
55 yeast, most of the secreted IDP and proteins exhibiting Intrinsically Disordered Regions (IDR)
56 are *O*-mannosylated. In bacteria, the specific need for α -mannose in such structure does not
57 seem essential since their intrinsic high content of proline favors extended conformation of
58 IDR. Still, elongation and further stiffening are observed upon glycosylation of these structures
59 [3].

60 In recent years there has been a growing interest towards glycosylation in bacteria.
61 Although glycosylation is not restricted to pathogenic bacteria, most studies were performed
62 in these bacteria and conclude to an important role of glycosylation on pathogenicity [1].
63 Indeed, this phenomenon was described in *Mycobacterium tuberculosis* [4] where the authors
64 demonstrated that disruption of the *M. tuberculosis* Rv1002c gene encoding the membrane-
65 associated protein *O*-mannosyl transferase (PMT) resulted in strong attenuation of the strain.
66 In *M. tuberculosis*, PMT is unique and shown to be responsible for the transfer of mannose
67 residues from polyprenol monophosphate mannose (PPM) to dedicated proteins [5]. In fact,
68 various putatively mannosylated proteins were identified in *M. tuberculosis* by Concanavalin
69 A affinity capture experiments [6]. Several lipoproteins including lipoproteins belonging to the
70 Llp family (Lipid binding lipoprotein) were identified among these proteins including LppX-tb.
71 The gene encoding LppX-tb was initially identified in a transposition mutant of *M. tuberculosis*
72 with an attenuated phenotype [7]. In this mutant, the subcellular distribution of phthiocerol
73 dimycocerosates (DIM) was highly altered as compared to the wild type strain. DIMs are one
74 of the major lipidic virulence factor in pathogenic mycobacteria and their translocation at the
75 surface of the bacterial envelope, essential for the virulence of the strain, is proposed to be
76 mediated by LppX-tb. This hypothesis is quite convincingly corroborated by the structure of
77 LppX-tb which resemble a U-shaped β -half-barrel delimitating a large hydrophobic cavity able
78 to accommodate a DIM molecule [7]. Although supposed to be glycosylated, the complete
79 characterization of LppX-tb modifications has not been so far reported. Indeed, glycosylation
80 of LppX-tb was previously reported during heterologous expression in slow and fast-growing
81 mycobacteria *M. smegmatis*, *M. bovis* and *M. abscessus* [8–10]. In *M. smegmatis*
82 glycosylation was only suggested and not demonstrated. Whereas further results were
83 reported for the two other species for which 1 to 2 hexoses were found associated to the N-

84 terminal peptide of the protein. However the exact glycosylation site(s) was/were never
85 reported. Similar results have been reported in *Corynebacterium glutamicum*, a model host in
86 which LppX-tb was found to be heterogeneously glycosylated with 1 to 4 hexose(s) units in its
87 N-terminal peptide (aa₆ to aa₂₉) and where α -mannosidase deglycosylation experiments
88 suggested that LppX-tb is indeed mannosylated [11]. Interestingly LppX-tb glycosylation was
89 found to be differently affected by mutations in the lipoprotein post-translational
90 modification pathway in *C. glutamicum* [11,12]. These results enabled the authors to propose
91 that the N-acylation enzyme Lnt associates with PMT but that acylation (S- and N-acylation) is
92 not a prerequisite for glycosylation of LppX-tb in *C. glutamicum*.

93 As mannosylated LppX-tb is engaged in the virulence process of *M. tuberculosis* we
94 wondered if the attenuation phenotype observed in the PMT mutant [4] resulted from the
95 absence of glycosylation of LppX-tb. For this, we first identified S16 and T18 as the two sites
96 of LppX-tb mannosylation. We then undertook the construction of a variant where both
97 amino acids were modified in order to test the functionality of this LppX-tb variant. Both the
98 glycosylated and non-glycosylated forms of LppX-tb were characterized and found associated
99 to the capsule layer of *M. smegmatis*. In *M. tuberculosis* Mt103, complementation of the
100 knock-out mutant of *LppX-tb* either with a wild or mutated copy of the *LppX-tb* gene fully
101 restored the release of DIM into the culture medium suggesting that glycosylation of S16 and
102 T18 of LppX-tb is not necessary for DIM translocation.

103 **2. Materials and methods**

104 *2.1 Bacterial strains and growth conditions*

105 *C. glutamicum* ATCC13032 RES167 [13] and its derivative *pmt::Km^r*, were routinely
106 cultured on BHI medium (Difco) at 30°C.

107 *Mycobacterium smegmatis* MC²155 and *Mycobacterium tuberculosis* Mt103 were
108 grown in Middlebrook 7H9 broth (Fluka) supplemented with Middlebrook ADC enrichment
109 and 0.5% glycerol or BHI. Tween 80 (0.05 %) was added to liquid broth to avoid clumping
110 except for capsule analysis.

111 *2.2. Plasmids*

112 Plasmids pCGL482 [14], pVWEx2 [15], pMV261-Gm (derivative of pMV261) [16] and
113 pVV16 [17] were used as expression vectors in *C. glutamicum*, *M. smegmatis* and *M.*
114 *tuberculosis* respectively. When appropriate, antibiotics were added at the following
115 concentrations: kanamycin (Km) 25 µg. ml⁻¹, chloramphenicol (Cm) 6 µg. ml⁻¹ for *C.*
116 *glutamicum* or 30 µg. ml⁻¹ for *E. coli*, gentamycin (Gm) 10 µg. ml⁻¹, hygromycin (Hyg) 25 µg.
117 ml⁻¹ and tetracyclin 10 µg. ml⁻¹.

118 Cloning of Cg-*pmt* and Ms-*pmt* in *C. glutamicum* : The *pmt* genes of *C. glutamicum* Cg-
119 *pmt* (Cg1014 or NCgl0854) and *M. smegmatis* Ms-*pmt* (MsmeG_5447) [4] were cloned under
120 the tac promoter into the expression vector pVWEx2 [15]. The *pmt* genes were amplified from
121 the genomic DNA of *C. glutamicum* ATCC13032 or *M. smegmatis* mc²155 by PCR using the
122 primers DirCgPmt-*Xba*I and RevCgPmt6His-*Bgl*II or DirMsmPmt-*Xba*I and RevMsmPmt6His-
123 *Bam*HI respectively (Table 1). The PCR products were digested with *Xba*I and *Bgl*II or *Xba*I and

124 *Bam*HI and cloned into the *Xba*I- and *Bam*HI-digested pVWEx2 resulting in the *pmt*-expressing
125 plasmids pVWEx2-CgPmt-His and pVWEx2-MsPmt-His.

126 Cloning *lppX*-tb in *M. tuberculosis*: Cloning of *lppX*-tb and *lppX*-tb Δ S16 T18A in the
127 mycobacterial expression vector pVV16 was performed by PCR as described in Sulzenbacher
128 et al. [7] except for the DNA template. Here we used plasmid pMV261 *LppX*-tb and pMV261
129 *LppX*-tb Δ S16 T18A as templates.

130 Transformation of the bacterial strains was performed by electrotransformation as
131 described for *E. coli* [18], *C. glutamicum* [19], *M. smegmatis* and *M. tuberculosis* [20].

132 2.3. *Pmt* gene disruption

133 The *pmt* gene (Cg1014 or NCgl0854) of *C. glutamicum* ATCC13032 was interrupted by
134 integration of non-replicative plasmid with a kanamycin resistance cassette into the 5' part of
135 the gene. Briefly, a 532 bp-DNA fragment of the *pmt* gene was amplified by PCR using primers
136 PMT1-3 and PMT1-4 (Table 1). The DNA fragment was cloned into the pPrime cloning vector
137 (5PRIME) and used to transform *E. coli* as described by the manufacturer. The recombinant
138 plasmid was transferred into *C. glutamicum* by electroporation and transformants were
139 selected on Km-containing plates. As the plasmid is unable to replicate into *C. glutamicum*,
140 only the bacteria with the plasmid integrated into the genome were able to grow in presence
141 of kanamycin. Correct integration of the plasmid into the *pmt* gene was checked by PCR
142 analysis on the genomic DNA using combinations of primers localized upstream and
143 downstream of the cloned 532 bp-DNA fragment (PMT1-1 and PMT1-6) and into the vector
144 (M13R and M13F(-20)). After sequencing of the PCR products, one clone (*pmt::Km^r*) was used
145 for further experiment.

146 2.4. Site directed mutagenesis of *LppX-tb*

147 Site-directed mutagenesis of the *lppX-tb* gene was performed by PCR-driven overlap
148 extension [21]. The mutagenic primers and flanking primers used to generate the mutated-
149 *lppX-tb* genes are listed below (Table 1). Plasmid pCGL482 [11] and pMV261 [8] both carrying
150 the native *LppX-tb* gene with a hemagglutinin (HA) and a His₆ epitope fused to the 3' part of
151 the gene were used as templates for PCR. The generated PCR fragments were digested with
152 *EcoRV-XhoI* or *EcoRI* and cloned either into the *EcoRV-XhoI* digested pCGL482-AmyE [11] for
153 *C. glutamicum* or the expression vector pMV261 for *M. smegmatis*. All the mutated genes
154 were sequenced to confirm the mutations. All mutated *lppX-tb* genes contained the
155 hemagglutinin and a His₆ epitope fused to their 3' part.

156 2.5. Purification of native *LppX-tb* and derivatives

157 Purification of *LppX-tb* and mutated *LppX-tb* from *C. glutamicum* was performed as
158 described in Mohiman et al. [11].

159 For purification from *M. smegmatis*, bacteria from liquid cultures were harvested and
160 resuspended in a 50 mM phosphate buffer pH 8.0 containing 4-(2-
161 Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma) as a protease inhibitor.
162 Cells were broken using a one shot cell disrupter (CellID) at 30kPsi. Unbroken cells were
163 removed by low speed centrifugation (4000 g for 15 min) and the cleared supernatant was
164 centrifuged at 106,000 g for 60 min (rotor Ti 50 Beckman). Total membranes were
165 homogenized in a 50 mM phosphate buffer pH 8.0 containing 4% LDAO (lauryl dimethylamine
166 oxide) and stirred 1h in order to efficiently solubilize membrane proteins. Insoluble material
167 was removed by centrifugation at 106,000 g for 60 min. The soluble membrane proteins were
168 diluted twice and NaCl and imidazole were added to final concentrations of 50 mM and 10

169 mM respectively in 25 mM phosphate buffer, 2% LDAO, pH 8.0. Ni-NTA agarose previously
170 equilibrated with 25 mM phosphate buffer pH 8.0 with 2% LDAO, 50 mM NaCl and 10 mM
171 imidazole was added to the soluble membrane proteins. The mixture was gently agitated for
172 1h at 4°C and loaded in an empty column. The agarose resin was washed with ten volumes of
173 25 mM phosphate buffer pH 8.0 with 1 % LDAO, 50 mM NaCl and 20 mM imidazole to remove
174 weakly associated proteins. LppX-tb was eluted with the same buffer containing 50 mM
175 imidazole. The eluted samples were precipitated with trichloroacetic acid (TCA) 10% to
176 concentrate the proteins. If necessary further purification of LppX-tb was performed with anti
177 HA agarose (Pierce) instead of TCA precipitation.

178 *2.6. Extraction and analysis of proteins from different cellular localisation in C. glutamicum*
179 *and M. smegmatis*

180 The proteins from the envelope and culture supernatant of *C. glutamicum* were
181 prepared as follows. After growth, cells were separated from the culture supernatant by
182 centrifugation at 7000 g for 10 min. Proteins from the culture supernatant (S) were
183 precipitated with 10% TCA (Sigma) at 4°C for 30 min. The pellet, recovered by centrifugation
184 (13 000 g, 10 min), was washed with cold acetone and resuspended in TE buffer pH8 (Tris-HCl
185 10 mM EDTA 1 mM). Extraction of envelope proteins (E) of *C. glutamicum* was performed with
186 SDS at high temperature. Briefly, the centrifuged cells were resuspended in Laemmli buffer
187 and after heating 5 min at 100°C, cells were discarded after centrifugation. The supernatant
188 containing the envelope proteins (E) and the proteins from the culture supernatant (S) were
189 analyzed on SDS PAGE.

190 Extraction and analysis of the proteins from the capsule of *M. smegmatis* was
191 performed as described by Sani et al. [22] using 1% Tween 80 and the proteins were
192 precipitated with TCA before analysis on SDS PAGE.

193 2.7. Western blotting

194 Protein extracts (5 to 10 µg of total protein) were separated by SDS-PAGE (12%) and
195 transferred to nitrocellulose membrane. Immunoblotting was performed using either anti-
196 His6, anti-HA- or Concanavalin A peroxidase-conjugates (Roche) as recommended by the
197 manufacturer. The blots were developed using clarity western ECL substrate (Bio-Rad). The
198 ImageQuant LAS 500 was used for chemiluminescence detection.

199 2.8. Mass spectrometry analysis

200 -Sample preparation-

201 LppX SDS-PAGE bands, corresponding to different strains, were excised from the gel
202 after Coomassie Blue staining. In-gel enzymatic digestion was performed using standard
203 conditions. Briefly, protein bands were extensively washed with acetonitrile (ACN) and 100
204 mM ammonium carbonate. The excised bands were treated with 10 mM dithiothreitol (DTT)
205 at 56 °C for 30 min. After DTT removal, cysteine carbamidomethylation was performed at
206 room temperature for 30 min by addition of 55 mM iodoacetamide. After removal of the
207 supernatant, the washing procedure was repeated, and gel slices were dried. Tryptic digestion
208 was performed overnight at room temperature by addition of 20 µl of 10 ng/µl Porcine Gold
209 Trypsin (Promega) diluted in 50 mM ammonium carbonate. Proteolytic peptides were
210 extracted first by addition of 20 µl of 50% ACN and 0.1% formic acid and second by addition
211 of 20 µl of 100% ACN. Tryptic peptides extracted were vacuum dried and resuspended in

212 0.05% ACN and 0.1% trifluoroacetic acid (TFA) prior to nano LC-MS/MS mass spectrometry
213 analysis.

214 **-Nano LC-MS/MS analysis-**

215 Nano LC-MS/MS analysis were performed with the triple-TOF 4600 mass spectrometer
216 (Absciex, Framingham, MA, USA) coupled to the Nano-RSLC system (Thermoscientific). Briefly,
217 peptides were desalted on a C18 reverse phase pre-column (C18 Acclaim Pepmap100, 3 μm ,
218 100 \AA , 75 μm i.d., 2 cm length) using a loading buffer containing H₂O/ACN/TFA
219 (98%/2%/0.05%) at 5 $\mu\text{l}/\text{min}$ and were then eluted at a flow rate of 300 nl/min from the
220 reverse phase analytical C18 column (C18 Acclaim Pepmap100, 2 μm , 100 \AA , 75 μm i.d., 50 cm
221 length) using a 5-35% solvent B gradient for 40 min. Solvent B was 0.1% formic acid in 100%
222 ACN and solvent A was 0.1% formic acid in water. Nano LC-MS/MS experiments were
223 conducted in Data Dependent acquisition method by selecting the 20 most intensens
224 precursors for CID fragmentation with Q1 quadrupole set at low resolution for better
225 sensitivity and with a collision energy ramp ($\pm 15\%$) set to 35 V.

226 **-Data analysis-**

227 Raw data were processed using MS data converter software (Absciex) and protein
228 identification was performed using the MASCOT search engine (Matrix science, London, UK)
229 against LppX sequences with carbamidomethylation of cysteines set as fixed modification.
230 Oxydation of methionines and glycosylation of serine and threonine were set as variable
231 modifications. Peptide and fragment tolerance were respectively set at 25 ppm and 0.05 Da.
232 Only peptides with mascot ions score higher than the identity threshold (30) at less than 1%
233 of false positive discovery rate are considered. For relative quantification of the LppX P₆₋₂₉

234 glycoforms, MS extracted ion chromatogram (XIC) were realized on LppX P₆₋₂₉ harboring
235 glycosylation using peakview software 1.2 (Absciex).

236 *2.9. Lipids extraction from culture supernatants, cell surface and cells of M. tuberculosis*

237 Culture supernatants (50 ml) were sterilized by filtration, lyophilized and lipids were
238 extracted following Bligh and Dyer protocol [23] and analyzed by LC/MS. Cells obtained from
239 100 ml cultures were extracted with 3 ml of water-saturated butanol for 16h. The butanol
240 extracts were collected and filter sterilized. The remaining cell pellets were extracted with
241 chloroform: methanol (1:2, v:v) once and chloroform: methanol (2:1, v:v) twice. The combined
242 extracts were subjected to Folch wash, dried and analyzed by LC/MS. LC/MS was performed
243 using an electrospray ionization/atmospheric pressure chemical ionization source in the
244 positive mode on an Agilent 6220 time-of-flight mass spectrometer equipped with a
245 MultiMode Source.

246

247 **3. Results**

248 *3.1. Identification of the glycosylation sites of LppX-tb in C. glutamicum*

249 In a previous study, we reported that LppX-tb was O-glycosylated in *C. glutamicum*
250 [11]. The glycosylation sites were located within the N-terminal part of LppX-tb between
251 amino acids 6 and 29 (Fig. 1). MS analysis identified heterogeneously glycosylated forms of
252 LppX-tb carrying from 1 to 4 hexose units. In this study, we tried to identify the glycosylation
253 site(s) within the peptide using conventional CID MS/MS techniques but unfortunately this
254 was not successful. To determine the residues targeted by glycosylation in LppX-tb we thus
255 performed a PCR-mediated approach to generate site-directed mutagenesis of putative
256 glycosylation sites within the N-terminal part of LppX-tb. In this part of the polypeptidic chain,
257 3 hydroxyl-bearing amino acids were present (serine S16 and S20, threonine T18). All were
258 predicted as putative glycosylation sites (Fig. 1) by the NetOGlyc 4.0 software [24]. Threonine
259 T18 and serine S20 were individually substituted by an alanine leading to the mutated-genes
260 LppX-tb T18A and LppX-tb S20A. As we failed in replacing serine S16 by alanine, this serine
261 was deleted leading to the mutated-gene LppX-tb Δ S16. All mutated-genes were generated
262 by PCR and cloned into the *C. glutamicum* expression vector pCGL482 under the control of the
263 amyE promoter [11]. Recombinant strains were grown in BHI at 30°C overnight. After growth,
264 cells were separated from the media by centrifugation and broken. Unbroken cells were
265 removed and total membranes (inner and outer membranes) were recovered by high speed
266 centrifugation. The membranes were solubilized in LDAO and solubilized membrane protein
267 extracts were loaded on Ni-NTA column to purify the native and mutated His tagged LppX-tb.
268 The purified proteins were analyzed by MS.

269 Different glycoforms of the N-terminal peptide were detected after proteolysis of the
270 native LppX-tb protein (Fig. 2A): the peptide with no modification (P₆₋₂₉), the peptide with 1,
271 2, 3 and 4 hexose units (P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉ Hex3 and P₆₋₂₉ Hex4). The P₆₋₂₉ Hex2 form
272 was the most abundant form detected (Fig. 2B). Analysis of the mutants shows no difference
273 in the glycosylated pattern between the native LppX-tb and LppX-tb S20A. For the mutants
274 LppX-tb Δ S16 and T18A, only P₆₋₂₉ and P₆₋₂₉ Hex2 were detected. A double-mutated LppX-tb
275 gene Δ S16-T18A was finally constructed and did not reveal any glycosylated peptide (Fig. 2C).
276 All these results demonstrate that S16 and T18 are both native glycosylation sites and that
277 each site is diglycosylated in the native protein. It is interesting to note that the glycosylation
278 of S16 and T18 was not depending on each other. Moreover, despite modification of the
279 glycosylation pattern, LppX-tb variants Δ S16, T18A and Δ S16-T18A were still membrane-
280 associated.

281 3.2. *Cg_PMT* is essential for glycosylation of LppX-tb in *C. glutamicum*

282 As seen previously, LppX-tb is O-glycosylated with hexoses but the exact chemical
283 nature of the modification was not determined. Nevertheless alpha mannosidase
284 deglycosylation experiment suggests that LppX-tb is indeed mannosylated in *C. glutamicum*
285 [11]. A similar result is suggested in *M. tuberculosis* since LppX-tb was detected among the
286 ConA affinity capture glycoproteome [6].

287 In *C. glutamicum*, different proteins were identified as glycosyltransferases. Among
288 these proteins, Pmt (encoding gene NCgl0854) was identified as a protein O-mannosyl
289 transferase which transfers mannose residues from lipid carriers to proteins [25]. To
290 investigate the possible role of Pmt in the glycosylation process of LppX-tb, a mutant of *pmt*
291 was constructed in *C. glutamicum*. The *pmt* mutant (*pmt::Km'*) was generated by integration

292 of a kanamycin resistance cassette into the 5' part of the gene and the broad glycosylation
293 protein pattern of the *pmt* mutant was tested. The wild type and *pmt::Km^r* *C. glutamicum*
294 strains were grown in BHI. The cells and culture supernatant were separated by centrifugation.
295 Treatment of whole cells with high concentrations of SDS is known to release cell wall
296 components without affecting cell integrity in *Corynebacterium*. This method was used to
297 extract proteins from the envelope. Proteins from the culture supernatant were recovered by
298 TCA precipitation. Both proteins from the envelope and culture supernatant were transferred
299 on SDS-PAGE and analyzed by immunoblotting with Concanavalin A peroxidase conjugate. As
300 seen in Fig. 3A, several glycoproteins were detected both in the envelope and supernatant
301 culture of the wild type strain. As expected no ConA reactive glycoproteins were detected in
302 the *pmt::Km^r* strain. The *pmt* gene of *C. glutamicum* (*Cg-pmt*) was cloned in the expressing
303 vector pVWEx2 under the control of the tac promoter and the recombinant plasmid was
304 transferred in the *pmt* mutant for complementation studies. When the wild type *pmt* gene is
305 expressed in *pmt::Km^r*, glycoproteins are again detected with a similar pattern to the wild type
306 strain whether IPTG was added or not. As described in Mahne et al [25], our results confirm
307 that Pmt is responsible for the glycosylation of secreted proteins in the culture supernatants.
308 In addition, in this study we detected other *pmt*-dependent glycosylated proteins associated
309 to the cell envelope of *C. glutamicum*.

310 As the *pmt::Km^r* mutant was confirmed, we specifically checked if glycosylation of
311 LppX-tb was dependent of this gene by expressing LppX-tb in the mutant *pmt*. The LppX-tb
312 gene was cloned in the expression vector pCGL482 and used to transform the wild type and
313 *pmt::Km^r* mutant of *C. glutamicum*. LppX-tb was partially purified from the membranes of
314 these strains and analyzed by SDS PAGE and western immunoblotting (Fig. 3B). As seen in Fig.
315 3B, a major protein of 25kDa was detected by Concanavalin A in the wild type strain. In the

316 mutant *pmt::Km^r*, a major band was detected slightly below than in the wild type strain and
317 was not detected by the Concanavalin A conjugate suggesting that LppX-tb is not glycosylated
318 in the *pmt::Km^r* mutant. To confirm that this is not related to a polar effect of the mutation,
319 the *pmt* gene of *C. glutamicum* *Cg-pmt* cloned into the expression vector pVWEx2 was co
320 expressed with LppX-tb in *pmt::Km^r*. In these conditions the migration behavior of LppX-tb
321 was restored. The corresponding band was detected by Concanavalin A whether IPTG was
322 added or not. Glycosylation of LppX-tb in the complemented strain was definitively confirmed
323 by MS analysis (Suppl. Fig. S1). As for the wild type, different glycoforms of the peptide were
324 detected (P₆₋₂₉ Hex2 and P₆₋₂₉ Hex4) as well as the 262 Da modification previously seen in LppX-
325 tb by Mohiman et al. [11]. In the mutant *pmt::Km^r* MS analysis confirmed that the peptide P6-
326 29 was not glycosylated (Suppl. Fig. S1) which is in accordance with the absence of ConA
327 binding seen on western immunoblot. These results demonstrate unambiguously that
328 glycosylation of LppX-tb is dependent on *Cg-pmt* in *C. glutamicum*.

329 Complementation studies were also performed with the *M. smegmatis* *pmt* gene
330 (*Msmeg_5447*). The PMT proteins encoded by *Ms-pmt* from *M. smegmatis* or *Cg-pmt* from *C.*
331 *glutamicum* are quite similar with a sequence identity of 42% (Suppl Fig. S2). Moreover some
332 residues important for the catalytic activity [25] are conserved in both *Ms-pmt* and *Cg-pmt*.
333 Despite this similarity, the heterologous complementation of the *C. glutamicum* *pmt::Km^r*
334 mutant with *Ms-pmt* was unsuccessful. Neither the glycoproteins from the envelope and
335 supernatant culture nor LppX-tb were detected by Concanavalin A in the complemented strain
336 (Fig 3C and 3D). The absence of glycosylation of LppX-tb was also confirmed by MS analysis.
337 As the absence of glycosylation might be related to an expression problem, we tried to purify
338 the protein PMT expressed from *Ms-pmt* during heterologous expression. The same
339 experiment was performed with *Cg-pmt* as a positive control. In both cases, we were not able

340 to detect the PMT proteins. Although this result suggests that very low amounts of Cg-PMT,
341 is sufficient to allow complementation, it does not allow us to conclude for the absence of
342 complementation by *Ms-pmt*.

343 3.3. Glycosylation of LppX-tb in *M. smegmatis*

344 Here, before proceeding on the role of LppX glycosylation, it was important to check
345 whether LppX-tb glycosylation sites and pattern in *Mycobacteria* are the same as those found
346 heterogously in *C. glutamicum*. For this, we choose to work in *M. smegmatis*, a fast growing
347 species which is easily manipulated and in which several glycosylation studies have been
348 performed so far.

349 *M. smegmatis* strain MC²155 carrying pMV261-LppX-tb [8] was grown in BHI and LppX-
350 tb was purified as described in materials and methods. A similar experiment was performed
351 in parallel with *C. glutamicum* using pCgl482-LppX-tb. Both purified proteins were analyzed by
352 western immunoblot using Concanavalin A peroxidase conjugate. As expected, LppX-tb was
353 detected by the ConA conjugate when purified from *C. glutamicum*. Surprisingly, we were not
354 able to visualize any signal when LppX-tb was purified from *M. smegmatis* suggesting that
355 either LppX-tb is not mannosylated or that the glycosylation level of LppX-tb is not high
356 enough to allow detection. In order to discriminate between these hypothesis, LppX-tb was
357 analyzed by MS analysis. As shown in Fig. 4, LppX-tb purified from *M. smegmatis* was found
358 to be glycosylated on the N-terminal part of the protein. Several forms of peptide P₆₋₂₉ were
359 detected. The non-glycosylated form was the most abundant but small amounts of the Hex1
360 and Hex2 glycoforms were also detected. This low amount of glycoforms detected in *M.*
361 *smegmatis* may account for the absence of detection by Concanavalin A.

362 As LppX-tb was found glycosylated in *M. smegmatis*, we wanted to determine if the
363 glycosylation sites of LppX-tb in *M. smegmatis* were the same as in *C. glutamicum*. Mutations
364 of the putative glycosylation sites S16, T18 and S20 of LppX-tb were generated according to
365 the same procedure as for *C. glutamicum* except that the mutated genes were cloned in
366 pMV261 an expression vector for mycobacteria. The recombinant plasmids were introduced
367 into *M. smegmatis* MC²155 and the strains were grown in Middlebrook 7H9 broth
368 supplemented with Middlebrook ADC enrichment and 0.5% glycerol and Tween 80 (0.05 %).
369 The different LppX-tb proteins were purified from the membrane fraction as described in
370 materials and methods and analyzed by MS (Fig. 5). When expressed in *M. smegmatis*,
371 different glycoforms of the peptide (peptide with no modification P₆₋₂₉, peptide with 1 and 2
372 hexose units respectively P₆₋₂₉ Hex1 and P₆₋₂₉ Hex2) were detected with native LppX-tb as well
373 as with the variant LppX-tb S20A. For the variant LppX-tb Δ S16 and T18A, only P₆₋₂₉ and P₆₋₂₉
374 Hex1 were detected demonstrating that both S16 and T18 are glycosylation sites and that
375 each site can be monoglycosylated. This was confirmed by analysis of the double mutant LppX-
376 tb Δ S16 T18A in which no glycosylated peptide could be found. As for *C. glutamicum* it is
377 interesting to note that the glycosylation of S16 and T18 was not depending on each other.
378 These results show that the residues targeted by glycosylation in LppX-tb are identical in both
379 *C. glutamicum* and *M. smegmatis*. This result is in line with a recent work supporting similar
380 process of glycosylation in *M. smegmatis* and *M. tuberculosis* [26].

381 3.4. Native and Δ S16 T18A variant of LppX-tb are associated to the outerlayer of *M. smegmatis*

382 In our work, when expressed in *M. smegmatis*, LppX-tb was detected in the membrane
383 fraction. In *M. tuberculosis* and *M. bovis* LppX-tb and its ortholog were found associated to
384 the outermost layers of the cells that is the cell wall [27–29] and the cell surface [27]. In these

385 mycobacteria, the cell wall is surrounded by a capsular layer [22]). We decided to analyze the
386 mycobacterial capsular layer of *M. smegmatis* to see if LppX could also reach this outerlayer
387 and whether S16 and T18 glycosylation was necessary for this localization.

388 The mycobacterial capsular layers of the different *M. smegmatis* strains MC²155 carrying
389 pMV261, pMV261-LppX-tb and pMV261 LppX-tb Δ S16 T18A were extracted from the cells as
390 described by Sani et al. [22]. For this experiment Tween 80 was not added in the culture media
391 since this detergent is known to shed the capsule into the medium. After extraction, the
392 proteins of the mycobacterial capsular layer were precipitated with TCA and analyzed by SDS
393 PAGE and immunoblotting (Fig. 6A). The non-recombinant strain was used as a negative
394 control. As seen in Fig. 6A similar amounts of proteins were extracted from the different
395 strains. According to the immunoblot, LppX was only detected in the outerlayer of both
396 recombinant strains whether S16 and T18 residues of LppX were modified or not.

397 As we noticed minor differences on SDS PAGE patterns of the different strains, an additional
398 capsular extraction was performed and the protein content of the mycobacterial capsular
399 layers were further analyzed on 8% SDS-PAGE (Fig. 6B). As seen in Fig. 6B, an additional
400 protein was specifically detected in the strain expressing the Δ S16 T18A variant of LppX-tb.
401 This protein was identified by MS as the 60-kDa chaperonin GroEL1 from *M. smegmatis* (Suppl
402 Fig. S3). Interestingly, the presence of GroEL1 in the capsule layer of *M. smegmatis* and *M.*
403 *tuberculosis* was previously reported [22]. Here we show that this protein is specifically
404 enriched in the capsule of *M. smegmatis* expressing the LppX-tb Δ S16 T18A variant and this
405 may suggest a specific function related to the absence of LppX glycosylation.

406 *3.5. The Δ S16 T18A variant of LppX-tb is still able to promote translocation of phthiocerol*
407 *dimycocerosates to the surface of M. tuberculosis*

408 In *M. tuberculosis* phthiocerol dimycocerosates are required for virulence of the
409 bacteria [7]. Since LppX-tb is necessary for translocation of these molecules to the surface of
410 *M. tuberculosis* and, because mannosylation was shown to contribute to *M. tuberculosis*
411 virulence, we reasoned that glycosylation of LppX-tb might be important for LppX-tb function.
412 As disruption of LppX-tb in *M. tuberculosis* had no effect for growth in 7H9 broth [7], this
413 mutant was chosen for complementation studies with the native and the double mutated ΔS_{16}
414 T_{18A} LppX-tb gene. As previously described, in contrast to *M. tuberculosis* wild type strain, the
415 LppX-tb null mutant fails to release DIM into the culture medium but retains the ability to
416 translocate these lipids across the plasma membrane [7]. We therefore decided to check the
417 repartition of DIM in various mutants: the LppX-tb knock-out strain and the knock-out strains
418 complemented with native or mutated LppX-tb cloned in the expression plasmid pVV16 (Fig.
419 7). For this experiment cells were grown in 7H9 medium containing ADC and 0.05% Tween 80
420 allowing the release of some outermost components of the cells in the culture media. In these
421 conditions DIM molecules were present in the cells of all strains and all strains produced
422 similar amounts of DIM (Fig. 7A). The chromatography profile of the DIM molecules was
423 however slightly different in the LppX-tb knock-out cells as compared to the other cells. As
424 expected and seen previously [7] DIM molecules were absent from the culture filtrates of the
425 knock-out strain. However DIM molecules were present in the culture filtrates of all
426 complemented strains including the strain harboring the double mutation $\Delta S_{16} T_{18A}$ (Fig. 7A).
427 This result demonstrates that the presence of DIM in the culture filtrate of *M. tuberculosis* is
428 not dependent on S16 and T18 LppX glycosylation of LppX-tb and therefore neither the
429 localization of DIM in the outerlayer of *M. tuberculosis*.

430 Further analysis of the composition of DIM was performed by LC/MS. As shown in Fig.
431 7B, cells of the knock-out strain mainly contain DIM A molecules whereas cells (Suppl. Fig. S4)

432 and culture filtrates of the two complemented mutants mainly contain DIM B molecules (Fig.
433 7B). Again the S16 and T18 LppX glycosylation state of LppX-tb has no impact in the
434 composition of DIM molecules.

435 4. Discussion

436 In this article we used heterologous hosts to determine the glycosylation sites of the
437 *M. tuberculosis* protein LppX-tb. A non glycosylated LppX-tb variant was then constructed and
438 used to study the impact of glycosylation on LppX-tb function in *M. tuberculosis*.

439 4.1. The glycosylation sites S16 and T18 are localized in the unfolded N-terminal part of LppX- 440 tb

441 No specific target sequon is known for *O*-glycosylation in contrast with *N*-glycosylation.
442 However *O*-glycosylation sites are most often localized within regions of low sequence
443 complexity [1]. Here in agreement with these studies we showed that LppX-tb glycosylation
444 sites are localized in the N-terminal region of the lipoprotein LppX-tb in a region lacking any
445 secondary structure [7]. Moreover as seen for other mannosylated mycobacterial proteins
446 [5,30] these modified sites are within a region enriched with amino acids proline and alanine.

447 Both S16 and T18 residues are also present in the corresponding LppX-tb ortholog of
448 *M. bovis* suggesting potential glycosylation at the same residues in this protein. In the *M.*
449 *leprae*, *M. marinum* *M. ulcerans* and *M. goodnae* LppX-tb-orthologs these residues differ at
450 position 16 and/or 18 but their N terminal regions are enriched with threonine and serine
451 residues with some of them predicted as potential glycosylation target (Suppl. Fig. S5).

452 4.2. The glycosylation sites S16 and T18 of LppX-tb are identical in *C. glutamicum* and *M.* 453 *smegmatis* but the glycosylated forms detected are different.

454 Whether LppX-tb is expressed in *C. glutamicum* or *M. smegmatis*, the lipoprotein is
455 found to be modified by a hexose (probably mannose) at exactly the same glycosylation sites.
456 However the glycosylation pattern differs between both bacteria. In *M. smegmatis* up to 2

457 hexoses are added to LppX-tb. The same result was reported during expression of LppX-tb in
458 other mycobacterium species *M. bovis* BCG [9] and *M. abscessus* [10]. In *C. glutamicum* up to
459 4 hexose residues were found associated to LppX-tb during expression together with an
460 unknown modification of 262 Da occasionally detected in the N-terminal peptide of LppX-tb
461 as seen previously [11]. This modification was never detected when LppX-tb was expressed in
462 *M. smegmatis*.

463 These results point out homologous sequence recognition in mycobacteria and
464 corynebacteria but also minor differences reflecting their specific metabolism. In
465 mycobacteria the highly glycosylated form of LppX-tb (Hex 3 and Hex 4) and the 262 Da
466 modification detected in *C. glutamicum* may either be absent and/or the amounts synthesized
467 not high enough to allow detection.

468 4.3. Glycosylation of LppX-tb is performed by Pmt

469 In *C. glutamicum* the gene *pmt* (Cg1014 or NCgl0854) encoding for a
470 mannosyltransferase responsible for protein-mannosylation was previously identified [25].
471 When expressed in the interrupted *pmt* mutant of *C. glutamicum*, LppX-tb is no longer
472 glycosylated. Glycosylation is recovered when complementation with the Cg-pmt gene is
473 performed. Thus our results confirm that Cg-pmt is involved in the glycosylation process of
474 LppX-tb in addition to the polyprenol phosphate mannose synthase (cg-ppm1) and the
475 apolipoprotein N-acyl transferase (cg-ppm2 or Int) in *C. glutamicum* [11]. Moreover as
476 previously proposed [11] mannosylation of LppX-tb is confirmed. The exact role of Cg-pmt
477 remains to be elucidated since Cg-pmt could be responsible both for the initial attachment of
478 the mannosyl residue to the target residues and the elongation of the oligosaccharide chain.

479 Alternatively other(s) glycosyltransferase(s) might be needed for the elongation step.
480 Previously identified glycosyltransferases in *C. glutamicum* [31] could be involved.

481 No complementation was noticed during heterologous expression of Ms-pmt of *M.*
482 *smegmatis* in the interrupted pmt mutant of *C. glutamicum*. This result was surprising since
483 we already observed successful complementation of *C. glutamicum* mutant with a *M.*
484 *smegmatis* gene [12]. However different hypothesis might explain this result. First during the
485 mannosylation process Pmt transfers mannose from polyprenol phosphate mannose (PPM)
486 to a target protein during its translocation across the membrane. In *M. tuberculosis* this
487 process requires interaction with the Sec complex [32]. Thus the interaction of Ms-pmt with
488 the Sec translocon of *C. glutamicum* might not be functional. Second the polyprenols involved
489 in mannose translocation in actinomycetales differ in chain length and saturation of the
490 isoprene unit [5]. Only C55-polyprenolmannose was detected in *C. glutamicum* [33] whereas
491 C35, C40 and C50-PPM were found in *M. smegmatis* [34]. The protein Ms-pmt may not be able
492 to recognize the C55-PPM of *C. glutamicum* and/or transfer the mannose. Finally the Ms-pmt
493 may also be not correctly expressed or localized in the cell.

494 According to our results (that is evidence of mannosylation of LppX-tb in different
495 actinobacteria and the role of Cg-Pmt) and considering that the proteins Pmt from *C.*
496 *glutamicum*, *M. smegmatis* et *M. tuberculosis* are homologous [4,25] Sm-Pmt and Mt-Pmt
497 are certainly responsible for LppX-tb mannosylation in the respective bacteria. Moreover as
498 the glycosylated residues of LppX-tb are the same in *C. glutamicum* and *M. smegmatis* we can
499 conclude that Cg-Pmt and Sm-Pmt are able to recognize identical glycosylation pattern in
500 LppX-tb. Although very likely, more experimental results are needed to expand this conclusion
501 to a larger group of proteins.

502 4.4. Distribution of DIM in the mycobacterial cell is not altered whether S16 and T18 residues
503 of LppX-tb are glycosylated or not

504 In lipoproteins, an intrinsically disordered domain is generally found between the N
505 terminal lipid anchor and the functional domain of the protein [35]. Various functions are
506 associated with intrinsically disordered proteins or regions such as connecting ordered
507 domains, regulation, molecular recognition and signaling and assisting in protein folding [3].
508 In lipoproteins this unfolded domain contains the lipoprotein sorting signal and is thought to
509 properly position the lipoprotein [35]. Moreover Prates et al. [3] proposed that the elongation
510 effect due to glycosylation of unfolded domains may be important to provide the optimum
511 distance between the connected domains for protein function. As the LppX-tb glycosylated
512 residues were positioned in this part of the lipoprotein, glycosylation could act as a signal for
513 its export and/or function. These data prompted us to evaluate the possible impact of LppX-
514 tb glycosylation on its localization and function. Our results show that the outerlayer
515 localization of WT and modified LppX-tb Δ S16T18A in *M. smegmatis* is identical. Moreover
516 LppX-tb harboring mutated S16 and T18 residues is still able to promote DIM translocation
517 across the cell envelope in *M. tuberculosis* suggesting that its function is not significantly
518 impaired. A similar result was also described for LprG, a structurally homolog protein of LppX-
519 tb. Indeed, LprG, which is O-mannosylated with a unique hexose on a threonine residue [26]
520 was also shown to be essential for correct surface localisation of complex lipids (mannose-
521 capped lipoarabinomannan (LAM)) in *M. tuberculosis* [36]. Interestingly, Alonso et al. showed
522 that the surface localization of LAM in a *pmt* mutant of *M. tuberculosis* is not impaired [26].
523 Thus it seems that the distribution of complex lipids such as LAM and DIM in the mycobacterial
524 cell is not dependent on the glycosylation state of their protein carriers LprG and LppX-tb

525 respectively. Consequently these results suggest that the absence of LppX and LprG
526 glycosylation does not account for the attenuation of virulence observed in the pmt mutant.

527 *4.5. Is there a role for GroEL1 in DIM translocation?*

528 In absence of mannosylation the function of LppX is not affected which is quite
529 surprising as generally glycosylation is thought to affect protein folding and stability. However
530 one can imagine that in the absence of glycosylation other factors may substitute for assisting
531 LppX stability to maintain a sufficient level of DIM translocation at the cell surface. In that
532 respect it is interesting to notice that Wang et al [37] previously discovered that the presence
533 of phthiocerol dimycocerosates in *M. bovis* BCG is dependent on GroEL1. Because we show
534 that the amount of GroEL1 is largely increased in the capsular layer of *M. smegmatis* only
535 when the non-glycosylated variant of LppX-tb is expressed, it is thus tempting to propose that
536 GroEL1 may be involved in the DIM translocation through an important role on LppX folding
537 and/or stability. Indeed in the absence of glycosylation, the N-terminal non ordered chain of
538 LppX may not be correctly extended and this effect could be reversed by GroEL1
539 overexpression. By extension, in *M. tuberculosis*, overexpression of GroEL1 could also
540 counteract the absence of glycosylation of LppX-tb Δ S16-T18A and explain that DIM are still
541 translocated at the cell surface. Further experiments are needed to validate this attracting
542 hypothesis.

543 **Conflict of interest**

544 None declared

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665

666 **Legends to figures**

667 **Fig. 1. Amino acid sequence of the mature lipoprotein LppX.** Bold letters indicate the
668 glycosylated peptide P₆₋₂₉ identified by Mohiman et al. [11]. The HA and His₆ epitopes are
669 highlighted. The underlined amino acids were predicted as putative glycosylated site
670 (prediction with NetOGlyc 4.0 Server <http://www.cbs.dtu.dk> [24]).

671 **Fig. 2. (A)** Extracted ion chromatograms and **(B)** relative abundances of the LppX P₆₋₂₉
672 glycoforms observed during expression of wild type WT and mutated lppX Δ S16, -T18A and -
673 S20A genes in *C. glutamicum* (CG). **(C)** Extracted ion chromatogram of LppX P₆₋₂₉ glycoforms
674 observed during expression of the mutated lppX Δ S16 T18A genes in *C. glutamicum*. All strains
675 were grown in BHI. The different glycoforms detected are the peptide with no modification
676 (P₆₋₂₉), the peptide with 1, 2, 3 and 4 hexose units (respectively P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉
677 Hex3 and P₆₋₂₉ Hex4). The amino acid sequence of the wild type and mutated 6 – 29 peptides
678 are mentioned in the right upper part of the figures using a red letter for the substituted amino
679 acid and an asterisk for the deleted one.

680 **Fig. 3. Pmt dependent protein glycosylation in *C. glutamicum*.**

681 Western blot analysis of glycoproteins detected in the envelope (E) or in the culture
682 supernatant (S) of the wild type 13032 strain, *pmt::Km^r mutant* and *pmt::Km^r mutant*
683 complemented by *Cg-pmt* **(A)** or *Ms-pmt* **(C)**. Glycoproteins were detected using Concanavalin
684 A peroxidase conjugate. Partial purification of LppX from the wild type strain 13032, *pmt::Km^r*
685 mutant and *pmt::Km^r mutant* complemented by *Cg-pmt* **(B)** or *Ms-pmt* **(D)**. (Left) Protein
686 pattern on 12% SDS PAGE after Coomassie blue staining. (Right) Glycoprotein pattern after
687 Western immunoblot using Concanavalin A peroxidase conjugate.

688 For complementation studies cells were grown in presence (+) or absence (-) of IPTG.

689 **Fig. 4. (A)** Relative abundance of the LppX P₆₋₂₉ glycoforms observed during expression of the
690 wild type *lppX* gene (WT) in *C. glutamicum* (CG) and *M. smegmatis* (MS) grown in BHI. **(B)**
691 Extracted ion chromatogram of the LppX P₆₋₂₉ glycoforms detected in *M. smegmatis* (MS). The
692 different glycoforms detected are the peptide with no modification (P₆₋₂₉), the peptide with 1,
693 2, 3 and 4 hexose units (P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉ Hex3 and P₆₋₂₉ Hex4)

694 **Fig. 5.** Relative abundances of the LppX P₆₋₂₉ glycoforms observed during expression of WT
695 and mutated *lppX* Δ S16, -T18A, -S20A and Δ S16-T18A genes in *M. smegmatis* (MS) grown in
696 Middlebrook 7H9 broth supplemented with ADC, glycerol and Tween. The different
697 glycoforms detected are the peptide with no modification (P₆₋₂₉), the peptide with 1 and 2
698 hexose units (P₆₋₂₉ Hex1 and P₆₋₂₉ Hex2).

699 **Fig. 6. LppX is found in the outermost layer of *M. smegmatis*.** *M. smegmatis* Mc²155 carrying
700 pMV261, pMV261-LppX and pMV261-LppX Δ S₁₆ T₁₈A were grown in Middlebrook 7H9 broth
701 supplemented with 0.5% glycerol. Mycobacterial capsule proteins were extracted with Tween
702 as described by Sani et al. [22], separated on a 12% SDS-PAGE **(A)** stained with coomassie blue
703 (Left) and analyzed by western-immunoblot using anti HA peroxidase conjugate (Right).
704 Additional and refined analysis was performed on a 8% SDS-PAGE stained with coomassie blue
705 **(B)**. The additional protein identified by MS is indicated (★).

706 **Fig. 7. LC-MS analysis of the distribution of DIM in the cells (cells) and culture filtrates**
707 **(Culture filtrate).** **(A)** Extracted ion chromatograms of DIM molecules from the *lppX* knock-
708 out strain (KO) and the knock-out strains complemented with wild-type (WT) or mutated
709 (Δ S16-T18) *lppX*. DIM molecules are absent from the culture filtrate of the knock-out strain,
710 but present in the culture filtrates of both complemented mutant strains. **(B)** Mass spectra of
711 DIM molecules extracted from the cells of the knock-out mutant, and from the culture filtrates

712 of the mutant complemented with WT or mutated *lppX*. Note that the cells of the knock-out
713 strain contain mainly DIM A molecules whereas cells (Suppl. Fig. S4) and culture filtrates of
714 the two complemented mutants mainly contain DIM B molecules. DIM A molecules are
715 derivatives of DIM B molecules in which a keto group is replaced with a methoxy group at the
716 terminus of the molecules. The ¹²C isotope peaks characteristic of DIM A and DIM B molecules
717 are labeled with their observed *m/z* [M+NH₄]⁺ values and their respective number of carbon
718 atoms.

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723 **Tables**

724 **Table 1.** Primers used in this study for the cloning and disruption of *pmt* and obtention of the
725 mutated *lppX* genes. The mutagenic and flanking primers used to generate site directed
726 mutations in the *lppX* gene are indicated. The mutated *lppX* genes were cloned into the
727 expression vector pCGL482 and pMV261.

Gene or plasmid	Primers
Cg- <i>pmt</i> cloning	DirCgPmt- <i>Xba</i> I 5'- CGCTCTAGAGCATCTAGCTTCCCACAAATGC-3' RevCgPmt6His- <i>Bgl</i> II 5'-CGCAGATCTTTAATGATGATGATGATGATGGCGCCA GCTTGGAACC-3'
Ms- <i>pmt</i> cloning	DirMsmPmt- <i>Xba</i> I 5'- GCGTCTAGAGCCGAGTGTCACGACCGACAGC-3' RevMsmPmt6His- <i>Bam</i> HI 5'-CGCGGATCCTTAGTGATGGTGATGGTGATGGCG CCAGCTCGGCAACC-3'
<i>pmt</i> disruption	PMT1-1 5'-GCCAAAGTTCAAGTGG-3' PMT1-3 5'-TTACAGGTTTGAGCAGTGC-3' PMT1-4 5'-ATACACCTGTGGTGAAAC-3' PMT1-6 5'-AAGAGCTGCAGGATGGAGTC-3' M13R 5'- CAGGAAACAGCTATGAC-3' M13F(-20) 5'- GTAAAACGACGGCCAG-3'
Mutated <i>lppX</i> gene :	Mutagenic primers
<i>lppX</i> ΔS ₁₆	Rev LppX S16delta 5'-GGGTCGGACGCCGTCGGCACGGGAACACCCTGTTCC-3' Dir LppX S16delta 5'-GGAACAGGGTGTTCCTGCGGACGGCGTCCGACCC-3'
<i>lppX</i> T _{18A}	Rev LppX T18A 5'-CGGGGTCGGACGCCGcCGGGCTCACGGG-3' Dir LppX T18A 5'-CCCGTGAGCCCGGCGGCGTCCGACCCCG-3'
<i>lppX</i> S _{20A}	Rev lppx-S20A 5'- CACTCGGGCTGCCCGGCTGGGGCGCGAGGAGC-3' Dir LppX S20A 5'- GTGAGCCCGACGGCGGCGGACCCCGCGCTCCTCG-3'
<i>lppX</i> ΔS ₁₆ -T _{18A}	RevS16deltaT18A 5'-CGGGGTCGGACGCCGcCGGCACGGGAACACC-3' Dir S16 deltaT18A 5'-GGTGTTCCTGCGGCGTCCGACCCCG-3'
Cloning plasmid	Flanking primers
pMV261	Dir pMV261 seq 5'- GGAATCACTTCGCAATGGCC- 3'

Rev LppX *EcoRI* 5'-CCACCACCATCACTAGAATTCGAGC-3'

pCGL482

482-S2 : 5'- GCAGAATAAATGATCCGTCG-3'

482-AS: 5'- GATATGATCATTATTCTGC-3'

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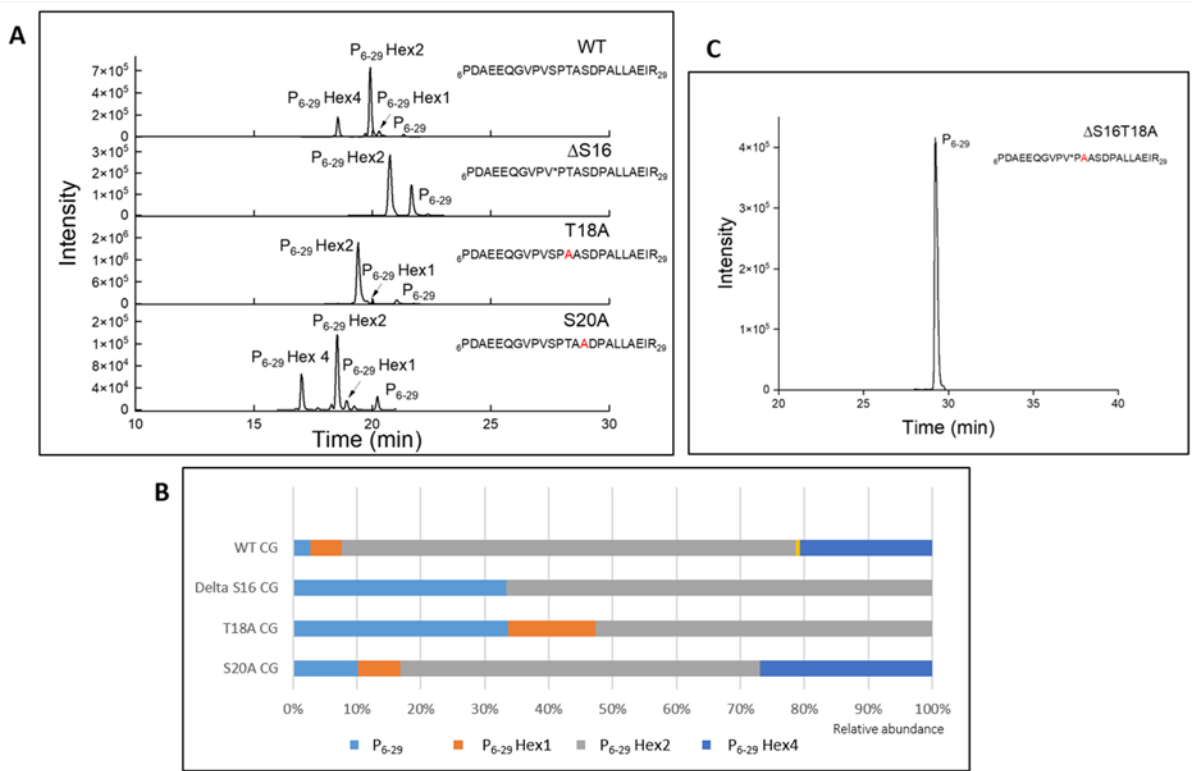
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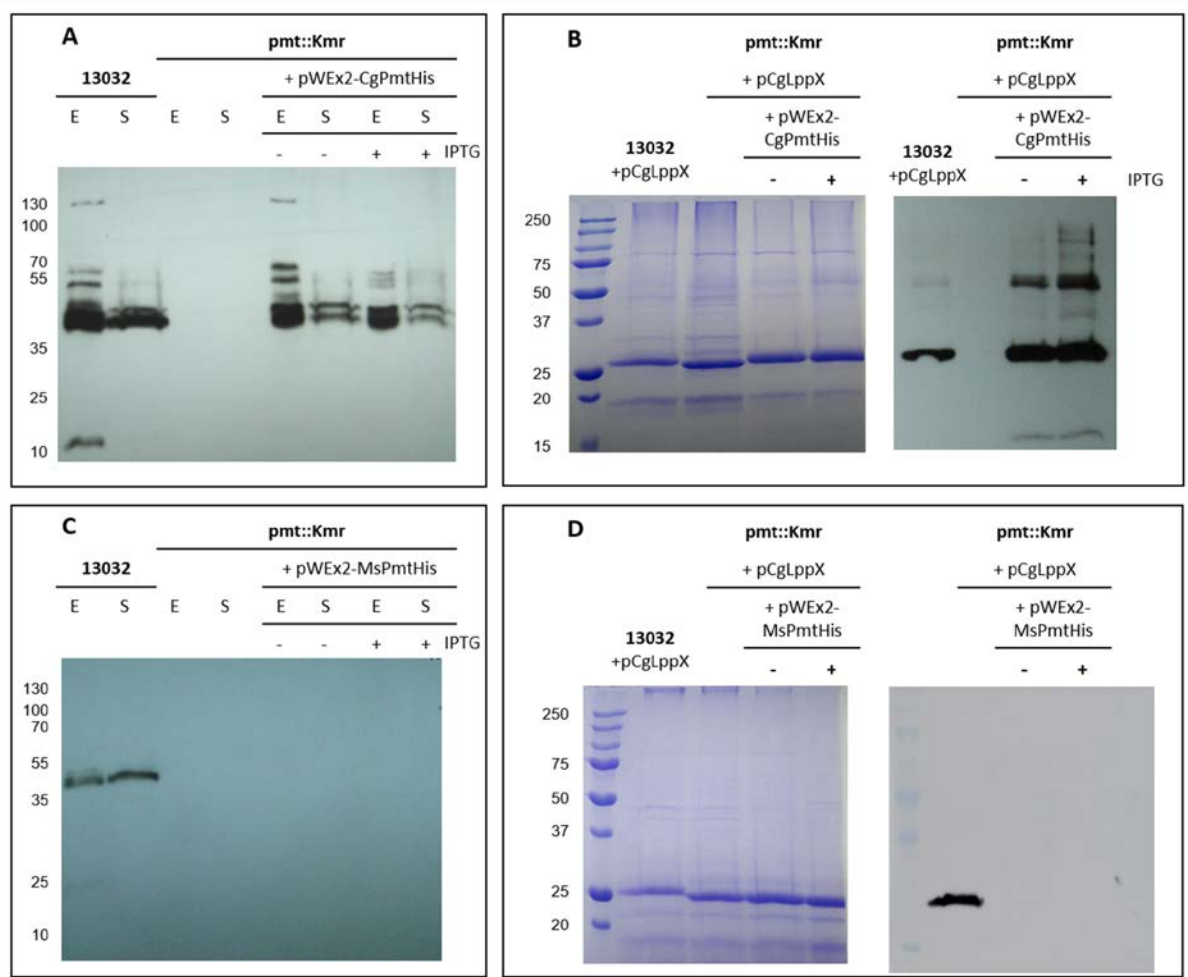
730 Fig. 1

CSSPKDAEEQGVPVSPTASDPALLAEIRQSLDATKGLTSVHVAVRRTTGKVDSLGITS
ADVDVRANPLAAKGVCTYNDEQGVFVRVQGDNISVKLFDDWSNLGSISELSTSRVL
DPAAGVTQLLSGVTNLQAQGTEVIDGISTTKITGTIPASSVKMLDPGAKSARPATVW
IAQDGSHHLVRASIDLGSGSIQLTQSKWNEPVNVDYPYDVPDYATWHHHHHH

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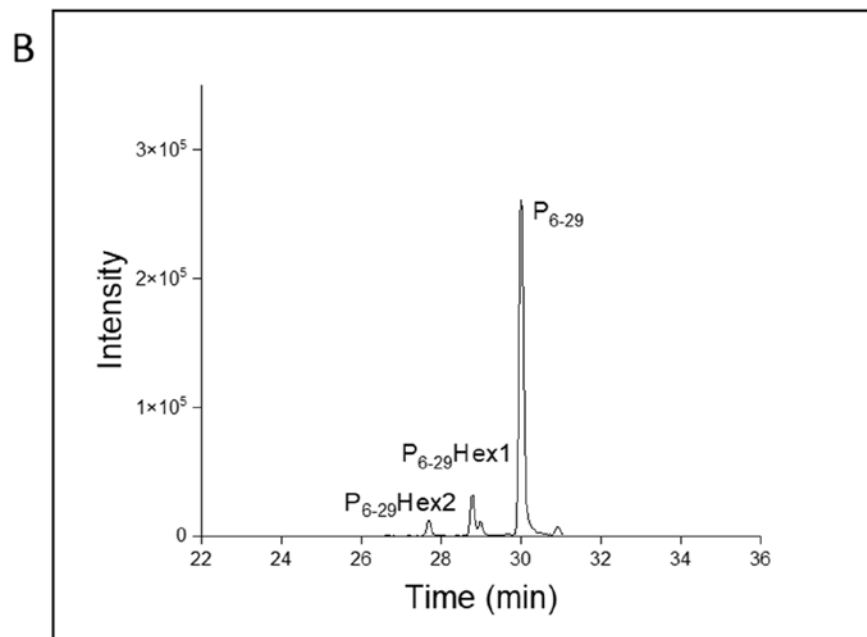
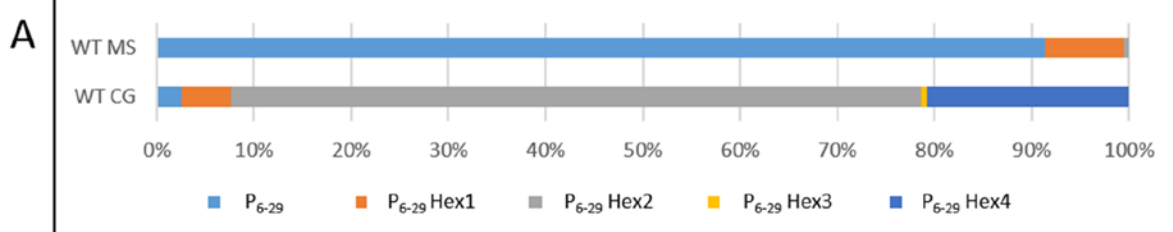




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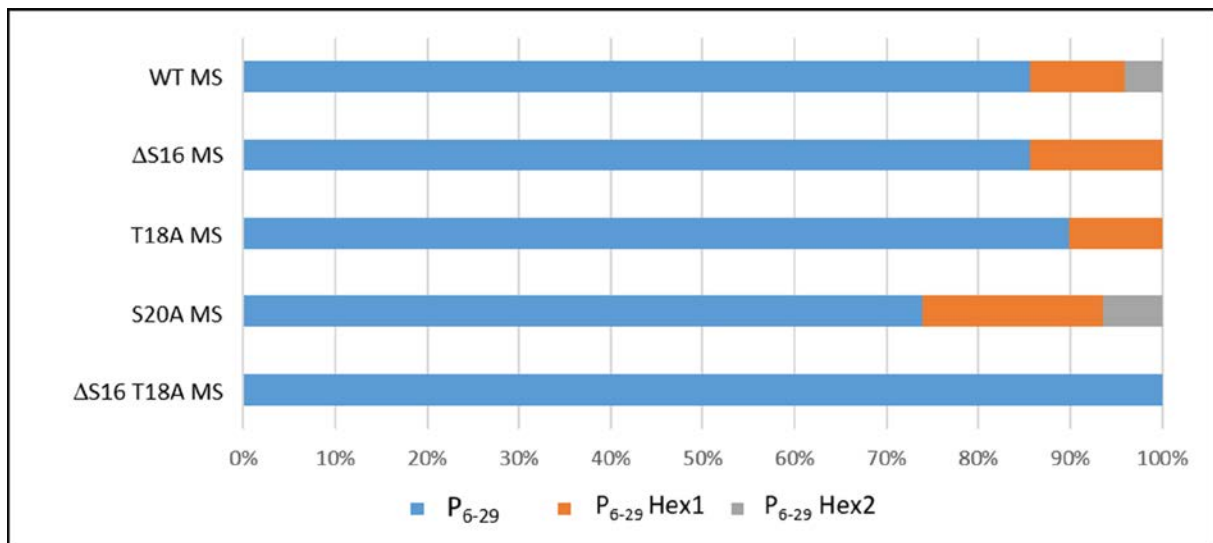
739 Fig.4



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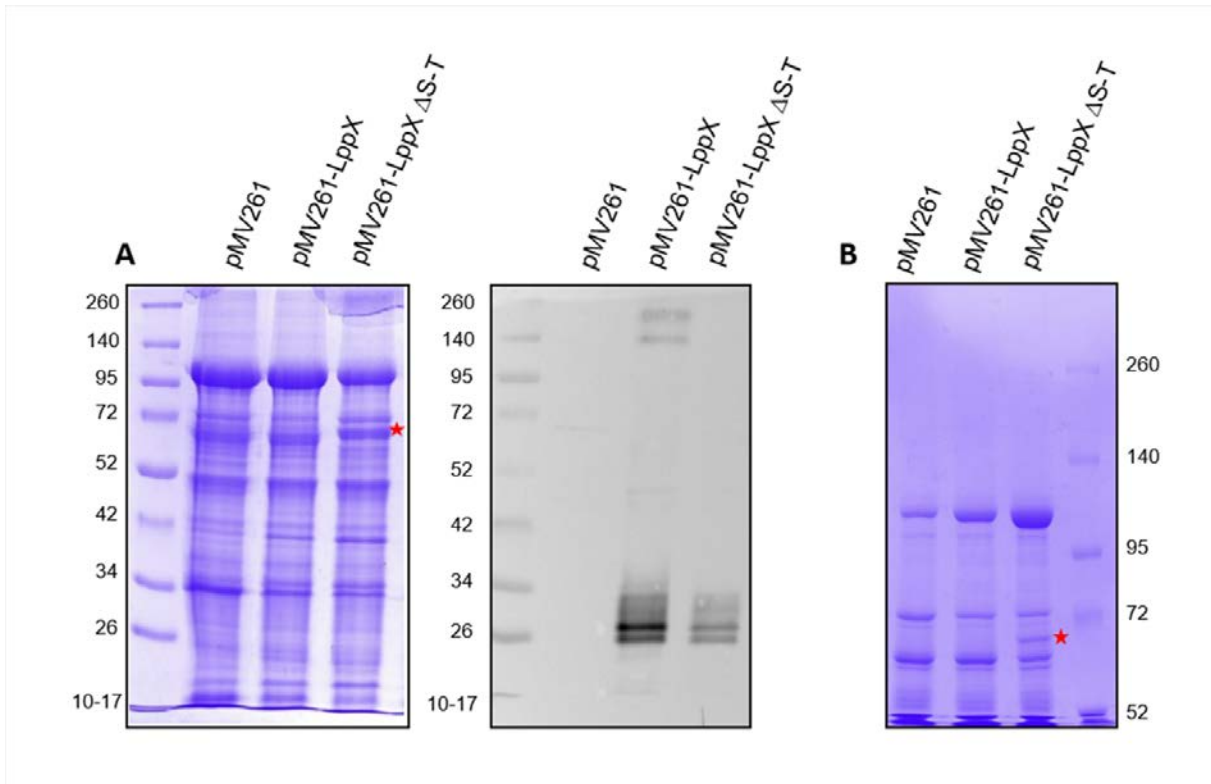
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742 Fig. 5



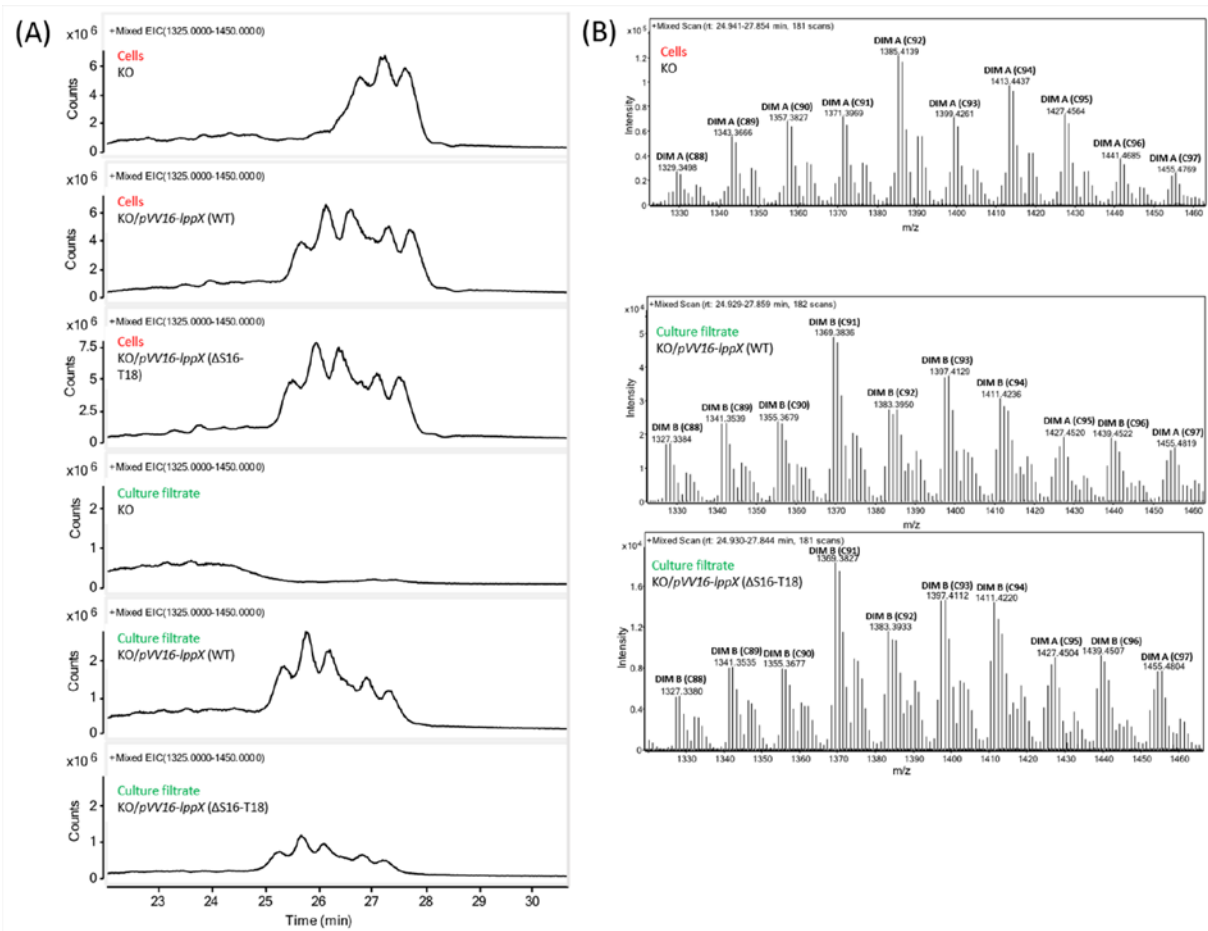
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751 **Supplementary material**

752 **Suppl Fig. S1. Complementation of the *pmt::Km^r* mutant of *C. glutamicum* with *Cg-pmt*.**

753 **(A)** Mass spectrum of the LppX₆₋₂₉ peptide and **(B)** relative abundances of the LppX P₆₋₂₉
754 glycoforms observed during expression of *lppX-tb* in the *pmt::Km^r* mutant complemented by
755 *Cg-pmt* of *C. glutamicum*. For the complementation studies cells were grown in presence (+)
756 or absence (-) of IPTG. **(C)** Extracted ion chromatograms of the LppX P₆₋₂₉ glycoforms observed
757 during expression of *lppX-tb* in the *C. glutamicum* wild-type strain (WT) and *pmt::Km^r* mutant.

758 **Suppl Fig. S2.** Sequence alignment of the PMT protein from *C. glutamicum* (PMT_Coryneglu)
759 and *M. smegmatis* (PMT_Mycosmeg) using EMBOSS Needle <https://www.ebi.ac.uk> [38]
760 (Identity : 228/539 (42.3%); Similarity : 310/539 (57.5%); Gaps : 42/539 (7.8%)). The conserved
761 residues seen in various PMT proteins [25] are framed in blue.

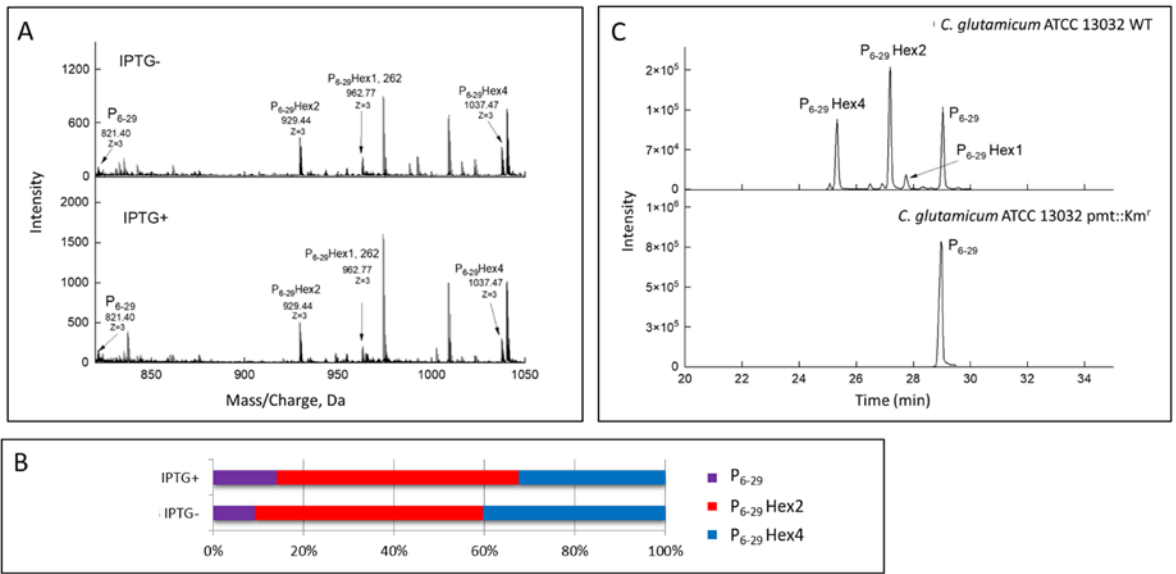
762 **Suppl Fig. S3. Mass spectrometry identification of the additional protein found in the**
763 **outerlayer of *M. smegmatis* MC²155 expressing the modified LppX-tb LppX Δ S₁₆ T₁₈A.**

764 Identified peptides **(A)** and sequence coverage **(B)** of the 60kDa Chaperonin 1
765 (*OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) OX=246196 GN=groL1 PE=1*
766 *SV=1*) resulting from in-gel trypsin digestion and Peptide-mass fingerprint (PMF) analysis.

767 **Suppl. Fig. S4.** Mass spectra of DIM molecules extracted from the cells of the *lppX* knock-out
768 mutant (KO) complemented with WT or mutated (Δ S16-T18) *lppX*. The ¹²C isotope peaks
769 characteristic of DIM A and DIM B molecules are labeled with their observed *m/z* [M+NH₄]⁺
770 values and their respective number of carbon atoms.

771 **Suppl. Fig. S5.** N- terminal predicted amino acid sequences of the mature LppX-tb orthologs
772 of *Mycobacterium bovis*, *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium*
773 *ulcerans* and *Mycobacterium gordonae*. The predicted O-glycosylation sites are indicated with

774 bold letters (prediction with NetOGlyc 4.0 Server <http://www.cbs.dtu.dk> [24] and the position
775 of the glycosylated amino acids 16 and 18 in LppX-tb are framed in blue.
776



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PMT_Coryneglu	1	-----MSQALPVRDQGRDQGIFAGTLPPAPPKFKWTRLD-TYTW-	38
PMT_Mycosmeg	1	MTALDTDPTAGRSAPL-----ISPGVIP-PPDF--GPLDRAQGWA	39
PMT_Coryneglu	39	--AIIAVFALVTRFTGLSSATASGTPVFDEKHYVPQAWDMVRSWINPITG	86
PMT_Mycosmeg	40	MTAIIITALAAITRFLNLGSPTDAGTPIFDEKHYAPQAWQVLHN-----D	83
PMT_Coryneglu	87	GIESNPGYGLVVHPP LAKQLEALGEWVFGYTPLGWRIMVAIFGTLTIFAI	136
PMT_Mycosmeg	84	GVEDNPGYGLVVHPPVVGKQLIAIGEWLFGYNGLGWRFSGAVCGVIVMLV	133
PMT_Coryneglu	137	MAIARRLSGSTMVTFIAGILALADGVLVSSRFGMLDIFLVFFITAAAWA	186
PMT_Mycosmeg	134	TRIARRISRSTLVGAIAGLLIIADGVSFVSSRTALLDVFLVMFAVAAFAC	183
PMT_Coryneglu	187	LIRDHQMQHQRNLNLLTNGQITKD-FGPRFGFRWWRFTTGVFLGLALS	235
PMT_Mycosmeg	184	LMVDRDQVRERMYHAFD-DGRIAETRWGTRLGVRWWRFGAGVLLGLACAT	232
PMT_Coryneglu	236	KWVGLYIIAFFGLTSVFLDLWLKRKRYGVRRYVTGTLKNDVIPALGSLVII	285
PMT_Mycosmeg	233	KWVGLYFVLFVGMVTLVFDIAIARKQYHVPHPWRGMLRRDLGPAAYVFLGI	282
PMT_Coryneglu	286	PALLYIWSRAWFASETSVYRHAKTDGTITEDSILQLFPESIAGWIHYHI	335
PMT_Mycosmeg	283	PFAVYLASYAPWFASETAVNRY-EVGRSIGPDSILPI-PDALRSLWHYTH	330
PMT_Coryneglu	336	SVLEFHGSLTSSGSHSPWDSKPWAWLVSGRPILYFSSTDISCDVGG---	382
PMT_Mycosmeg	331	AAVRFHNSLNTNADGNHHPWESKPWTWPMSLRPVLYAIDNQ---DVPGCCA	377
PMT_Coryneglu	383	-TCRMIYLFGTPAIWVLTVPVILWALWSFFARRSRGYVPLVFAAAGFL	431
PMT_Mycosmeg	378	QSCVKAVMLVGT PAMWFIAPVVLGWALWRTVVRDWRVYAVLVGYMAGFL	427
PMT_Coryneglu	432	PWLAAYDRQMYFFYATALVPFTIIMLALACGELWGRGKMTPTGLTRGSM	481
PMT_Mycosmeg	428	PWFADIDRQMYFFYATVMAFFVLVLAIALILGDILYKPNQNPERRTLGLLT	477
PMT_Coryneglu	482	VVTYISLVMMFLAFSPLFYGFVIPDYVYESLMWFPSWR 520	
PMT_Mycosmeg	478	VCFYVALVITNFAWMYPILTGLPISQTTWNLQIWLPSWR 516	

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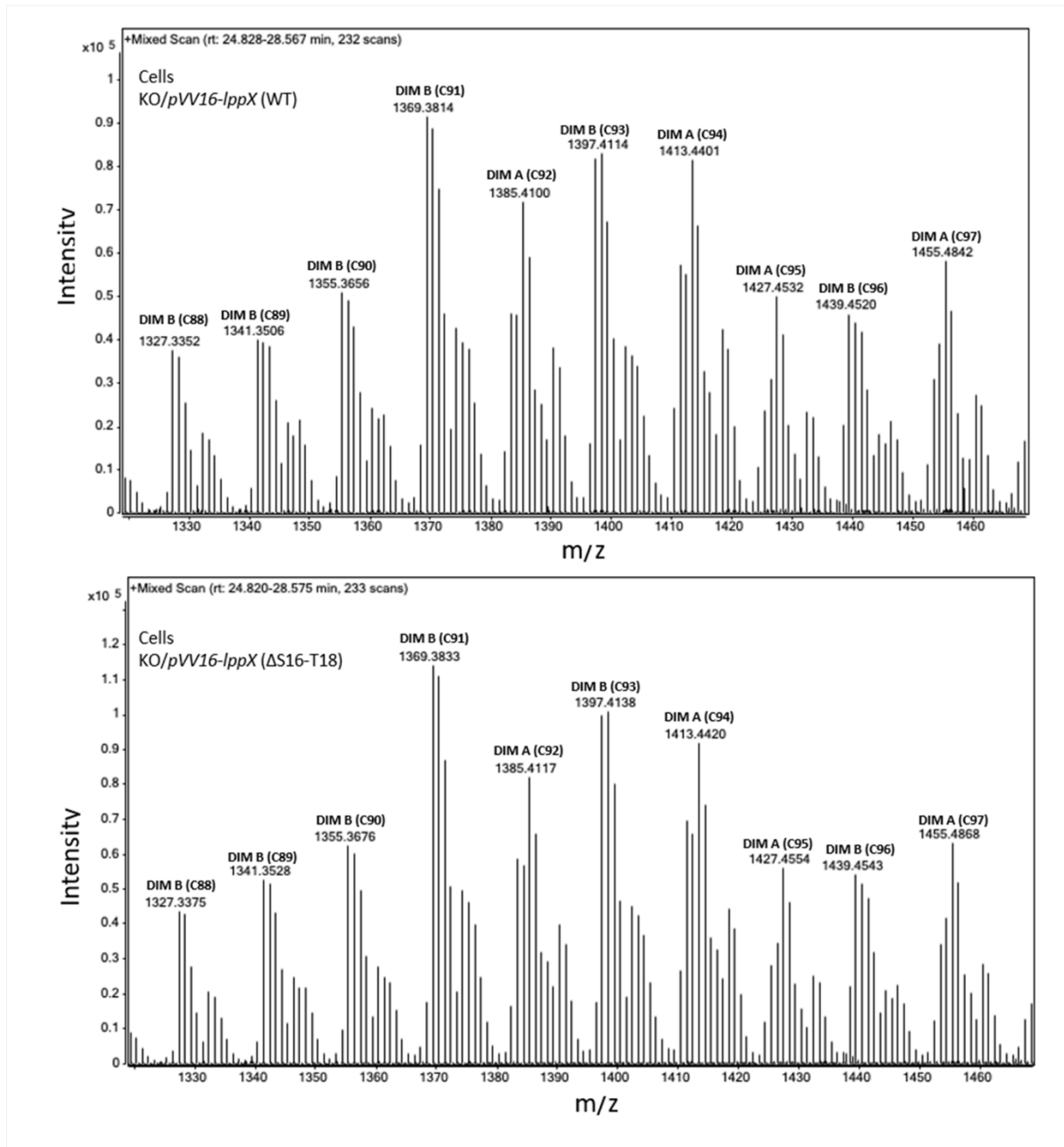
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A.						B.
Start - End	Observed	Mr (expt)	Mr (calc)	ppm	M	Peptide
4 - 12	1067.5026	1066.4953	1066.4931	2.11	0	K.TIAYDEEAR.R
4 - 13	1223.6019	1222.5946	1222.5942	0.37	1	K.TIAYDEEAR.G
18 - 27	987.5530	986.5458	986.5396	6.21	0	R.GLNSLADAVK.V
58 - 67	1264.5911	1263.5838	1263.5870	-2.53	0	K.EIELEDPEYK.I
79 - 100	2203.1602	2202.1529	2202.1492	1.68	1	K.KTDDVAGDGGTTATVLAQALVR.E
105 - 117	1280.7460	1279.7387	1279.7360	2.10	1	R.NVAAGANPLGLKR.G
196 - 208	1477.7025	1476.6952	1476.6885	4.55	0	K.GYISGYFVTDAR.Q
276 - 283	875.4441	874.4368	874.4409	-4.73	1	K.APGFGDRR.K
349 - 362	1670.7175	1669.7102	1669.7067	2.11	1	R.AEIEIENSDDSYDREK.L
379 - 390	1273.6810	1272.6737	1272.6673	5.01	1	K.AGAATEVELKER.K
392 - 399	995.5381	994.5308	994.5308	0.0070	1	K.HRIEDAVR.N

60 kDa chaperonin 1 OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) OX=246196 GN=groL1 PE=1 SV=1	
Protein sequence coverage: 22%	
Matched peptides shown in bold red .	
1	MAKTIAYDEE ARRGLERGLN SLADAVKVTL GFHGRNVVLE KHONGAPTIIN
51	DGVSIARIE LEDPYEKIGA ELVKEVAKKT DDVAGDGGTT ATVLAQALVR
101	EGLRWVAAGA NPLQLKRGIE KAVEKVTETL LKSAKEVETK EQIAATAGIS
151	AGDQSIGDLI REAMDKVQNE GVITVEESNT FGLQLELIEG HRFDRKQYISG
201	YFVTDARRQE AVLEDPYILL VSSKVSIVKD LLPLEKVIQ SGRPLLIIE
251	DVEGEALSTL VVNIKIGTFK SVAVKAPQPG DRRKAMLDQM AILTGGQVIS
301	EEVGLSLETA DVSLLGKARK VVTKDETTI VEGAGDAEAI QGRVAQIRAE
351	IENSDDSYDR EKLQERLAKL AGGVAVIKAG AATEVELKER KHRIEDAVRN
401	AKAAVEEGIV AGGGVALLQS AFSLEELSLT GDEATGANIV RVALSAPLKQ
451	IALNGGLEFG VVAEKVSNLP AGHGLNATG EYEDLLAAGV ADPVKVTIRSA
501	LQWASIAAL FLTTEAVVAD KFEKAAAPAG DFTGGGQMD F

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789 Suppl Fig. S5

<i>M. bovis</i>	SIU01591.1	CSSPKPDAEEQGVV SPTAS DPALLAEIRQSLDATKGLTS
<i>M. leprae</i>	WP_010907555.1	C S STKPDAQEQ SSSSS PASSDPAL T AEIKQSLETTKALSS
<i>M. marinum</i>	WP_094357885.1	CSSDKTQPEAQ SSTT VPADPALMAEIEQSLDATKALSAH
<i>M. ulcerans</i>	WP_134427244.1	CSSDKTQPEAQ SSTT VPADPALMAEIEQSLDATKALSAH
790 <i>M. goodsonae</i>	WP_069434779.1	CASAPKE S EAPPAT GTTTDS PLMADVRSV DATKALSAH

791